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13. ABSTRACT (Maximum 200 Words)

The purpose of this study is to test whether the greater extent of intracellular spreading of encoded antigen will generate a higher degree of antigen-specific immunity and ant-tumor effects in vaccinated mice. We previously showed that DNA vaccine of HVP22 linkage with to a model antigen, E7 generates strong CD8 specific T cell response and anti-tumor effects. We generated a DNA vaccines encoding BVP22 and MDV-1 VP22 linked with E7, respectively in this report. We demonstrated that compared with mice vaccinated with DNA encoding wild-type E7, mice vaccinated with BVP22/E7 and MDV-1 VP22/E7 DNA exhibited a significant increase in number of E7-specific $CD8^+$-T-cell precursors as well as stronger anti-tumor effects. Furthermore, our data indicated that the anti-tumor effect was CD8 dependent. These results suggested that the development of vaccines encoding VP22 fused to a target antigen might be a promising strategy for improving DNA vaccine potency. In addition, we have generated the pcDNA3-HVP22, pcDNA3-BVP22, pcDNA3-MVP22, pcDNA3-mesothelin, pcDNA3-HVP22/mesothelin, pcDNA3-BVP22/mesothelin and pcDNA3-MVP22/mesothelin for the control of ovarian cancer in task 1 of statement of work from months 0 to 9.

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INTRODUCTION

There is need to develop new therapies for ovarian cancer because current treatments for ovarian cancer, such as chemotherapy and radiation therapy, rarely result in a long-term cure for patients with local and metastatic disease. DNA vaccine represents a novel and attractive approach for cancer therapy. However, there are two major limitations of DNA vaccine potency. First, only a low percentage of professional antigen cell-dendritic cells within the skin actually uptake the DNA. Second, DNA vaccine is unable to amplify and spread in vivo as some viral vaccine vector is able to do. Recently, we have enhanced DNA vaccine potency using herpes simplex virus of HSV-1 VP22 (HSV-1) VP22, an HSV-1 tegument protein that demonstrated the remarkable property of intercellular transport and is capable of distributing proteins to surrounding cells [1]. Furthermore, the linkage of HSV-1 VP22 (HVP22) to E7 led to dramatic increase in the number of E7-specific CD8 T cell precursors in vaccinated mice (around 50-fold) and converted a less effective DNA vaccine to one with significant potency against E7-expressing tumors [2]. Recently, two additional examples of proteins with purported intercellular spreading properties have emerged, bovine herpes virus VP22 (BVP22) and Marek's disease virus VP22 (MVP22), both of which are VP22 homologues [3, 4]. Bovine herpes virus VP22 shares about 22% amino acid identify to human herpesvirus VP22. Marek's disease virus VP22 shares about 17% amino acid identity to HSV-1 VP22 and is also capable of intercellular transport after exogenous application [5]. The purpose of this study is to test whether the greater extent of intracellular spreading of encoded the ovarian cancer antigen mesothelin will generate a higher degree of antigen-specific immunity and ant-tumor effects in vaccinated mice.

BODY

We have demonstrated that DNA vaccine of HVP22 linkage with to a model antigen, human papillomavirus type 16 E7 generates strong CD8 specific T cell response and anti-tumor effects. If intercellular spreading strategy really facilitate the spreading of antigen encoded by the DNA vaccine to other dendritic cells and significantly increase the number of dendritic cells that are capable of stimulating tumor-specific T cells, we would anticipate that VP22 homologues of BVP22 and MDV-1 VP22 should have similar effects. We generated a DNA vaccines encoding BVP22 and MDV-1 VP22 linked with E7, respectively. We demonstrated that compared with mice vaccinated with DNA encoding wild-type E7, mice vaccinated with BVP22/E7 and MDV-1 VP22/E7 DNA exhibited a significant increase in number of gamma-interferon-secreting, E7-specific $CD8^+$-T-cell precursors (Figure 1 and 2) as well as stronger anti-tumor effects (Figure 3 and 4). Furthermore, our data indicated that the antitumor effect was CD8 dependent (Figure 5and 6). These results suggested that the development of vaccines encoding VP22 fused to a target antigen might be a promising strategy for improving DNA vaccine potency.

In addition, we have generated the pcDNA3-HVP22, pcDNA3-BVP22, pcDNA3-MVP22, pcDNA3-mesothelin, pcDNA3-HVP22/mesothelin, pcDNA3-BVP22/mesothelin and pcDNA3-MVP22/mesothelin for the control of ovarian cancer in task 1 of statement of work from months 0 to 9.

Figure 1

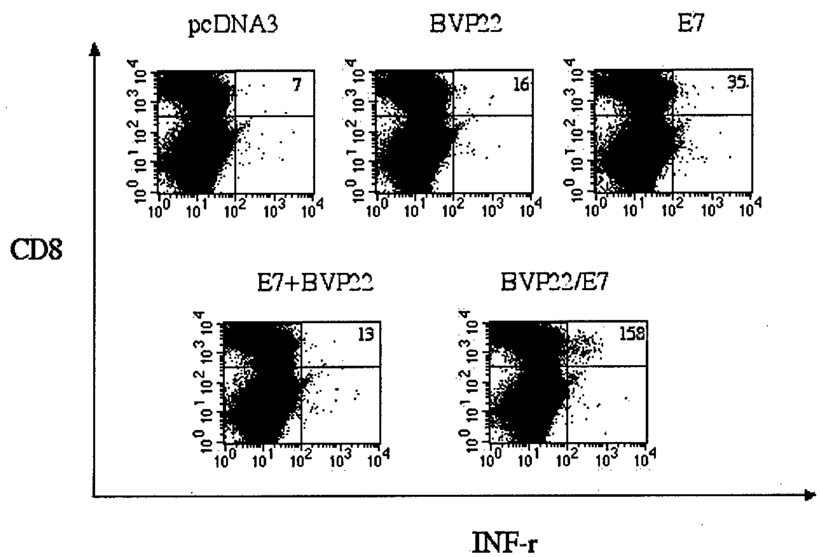

Figure 1. Immunologic responses generated by various recombinant DNA vaccines. (A) Flow cytometry analysis of IFN-γ-secreting E7-specific $CD8^+$ T cell precursors. Mice were immunized and splenocytes were collected and cultured. Note: Vaccination of mice with BVP22/E7 DNA generated the greatest number of IFN-γ $CD8^+$ T cell precursors compared to vaccination with the other constructs. The results of one representative experiment are shown out of three that were performed.

Figure 2

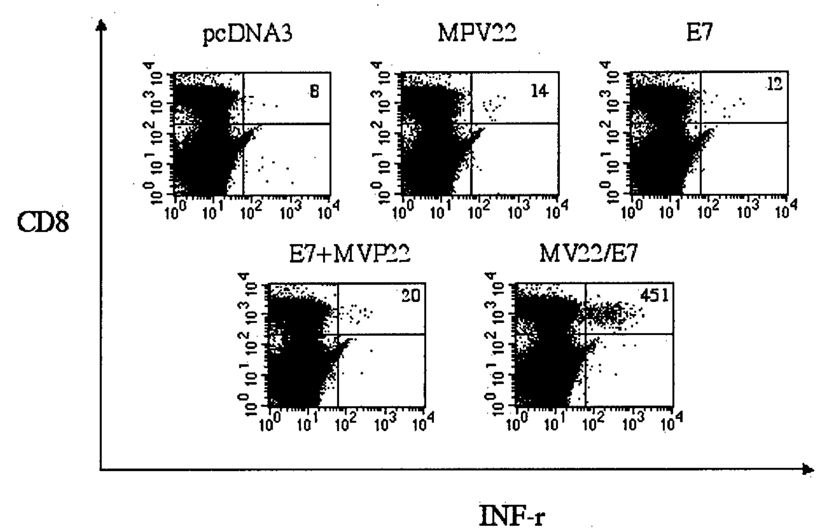

Figure 2. Immunologic responses generated by various recombinant DNA vaccines. (A) Flow cytometry analysis of IFN-γ-secreting E7-specific $CD8^+$ T cell precursors. Mice were immunized and splenocytes were collected and cultured. Note: Vaccination of mice with MVP22/E7 DNA generated the greatest number of IFN-γ $CD8^+$ T cell precursors compared to vaccination with the other constructs. The results of one representative experiment are shown out of three that were performed.

Figure 3

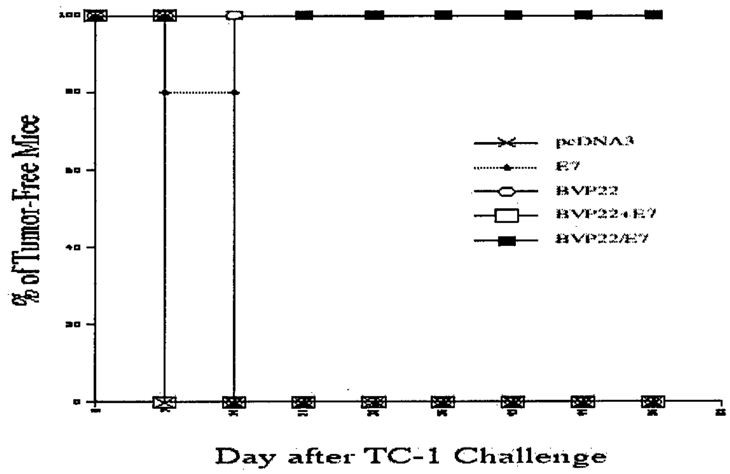

Figure 3. Tumor protection experiments and the effect of BVP22/E7 on subcutaneous tumor cell growth.
In vivo tumor protection experiment. For *in vivo* tumor protection experiments, mice were immunized and challenged subcuteneously with TC-1 tumor cells. The data collected from the *in vivo* tumor protection experiments shown here are from one representative experiment of two performed.

Figure 4

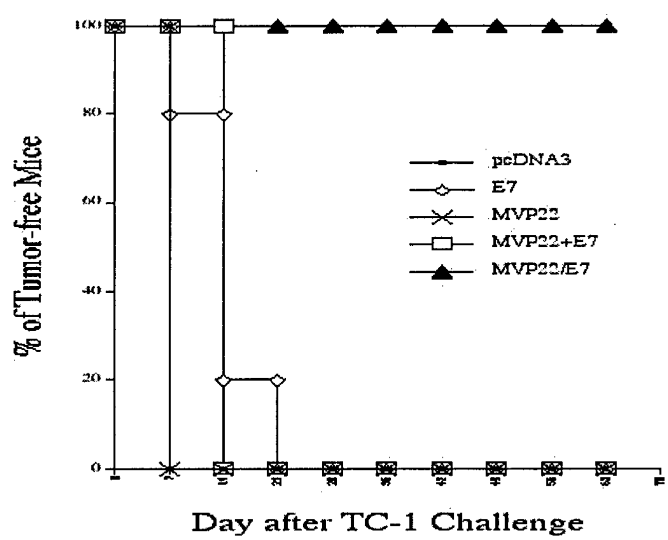

Figure 4. Tumor protection experiments and the effect of MVP22/E7 on subcutaneous tumor cell growt.
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Figure 5

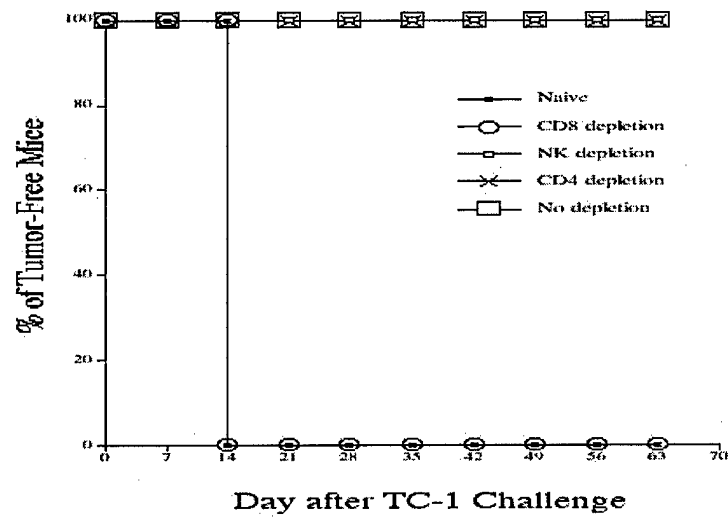

Figure 5. ***In vivo*** **antibody depletion experiment to determine the effect of lymphocyte subsets on antitumor effect.**
An *in vivo* antibody depletion experiment was performed to determine the effect of lymphocyte subsets on the potency of the BVP22/E7 DNA vaccine. Mice were vaccinated, tumor-challenged, and depleted of the relevant subset of lymphocytes.
Note: Only mice depleted of CD8 T cells, but not CD4 T cells or NK cells depleted mice, exhibited significant tumor growth.

Figure 6

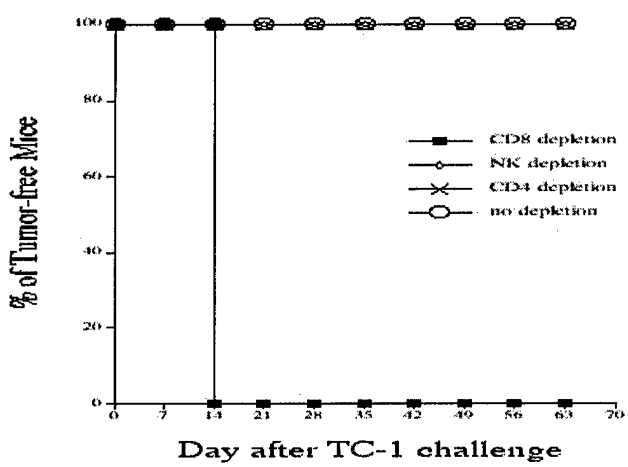

Figure 5. ***In vivo*** **antibody depletion experiment to determine the effect of lymphocyte subsets on antitumor effect.**
An *in vivo* antibody depletion experiment was performed to determine the effect of lymphocyte subsets on the potency of the MVP22/E7 DNA vaccine. Mice were vaccinated, tumor-challenged, and depleted of the relevant subset of lymphocytes.
Note: Only mice depleted of CD8 T cells, but not CD4 T cells or NK cells depleted mice, exhibited significant tumor growth.

KEY RESEARCH ACCOMPLISHMENTS:

♦ Mice vaccinated with BVP22/E7 and MDV-1 VP22/E7 DNA exhibited a significant increase in number of E7-specific $CD8^{+}$-T-cell precursors compared to mice vaccinated with E7

♦ Mice vaccinated with BVP22/E7 and MDV-1 VP22/E7 DNA showed stronger anti-tumor effects compared to mice vaccinated with E7

♦ Constructs of pcDNA3-HVP22, pcDNA3-BVP22, pcDNA3-MVP22, pcDNA3-mesothelin, pcDNA3-HVP22/mesothelin, pcDNA3-BVP22/mesothelin and pcDNA3-MVP22/mesothelin were done

REPORTABLE OUTCOMES:

For MDV-1 VP22/E7 data, we have published it in JOURNAL OF VIROLOGY, Mar. 2002, p. 2676–2682 [6]. Its title is "Improving DNA Vaccine Potency by Linking Marek's Disease Virus Type 1 VP22 to an Antigen".

CONCLUSIONS:

We demonstrated intercellular molecules of HVP22, BVP22, and MDV-1 VP22 linkage with model antigen E7 are able to induce strong immune response and anti-tumor effects. It suggests the intercellular spreading strategy is powerful tool to manipulate immune system to generate strong anti-tumor effects. This strategy is potentially used in other antigens such as ovarian cancer antigen, mesothelin.

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Project 3
Appendix 1

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Improving DNA Vaccine Potency by Linking Marek's Disease Virus Type 1 VP22 to an Antigen

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We have previously employed an intercellular spreading strategy using herpes simplex virus type 1 (HSV-1) VP22 protein to enhance DNA vaccine potency because DNA vaccines lack the intrinsic ability to amplify in cells. Recently, studies have demonstrated that the protein encoded by UL49 of Marek's disease virus type 1 (MDV-1) exhibits some degree of homology to the HSV-1 VP22 protein and features the property of intercellular transport. We therefore generated a DNA vaccine encoding MDV-1 VP22 linked to a model antigen, human papillomavirus type 16 E7. We demonstrated that compared with mice vaccinated with DNA encoding wild-type E7, mice vaccinated with MDV-1 VP22/E7 DNA exhibited a significant increase in number of gamma-interferon-secreting, E7-specific CD8$^+$-T-cell precursors as well as stronger tumor prevention and treatment effects. Furthermore, our data indicated that the antitumor effect was CD8 dependent. These results suggested that the development of vaccines encoding VP22 fused to a target antigen might be a promising strategy for improving DNA vaccine potency.

DNA vaccines have emerged as an attractive approach for generating antigen-specific immunotherapy (for reviews, see references 7, 26, 28, and 31). DNA is relatively stable and can be easily prepared and harvested in large quantities. In addition, naked plasmid DNA is relatively safe and can be administered repeatedly. Furthermore, because DNA can be maintained in cells to allow long-term expression of the encoded antigen, maintenance of immunologic memory is possible. Since naked DNA has no cell type specificity, it is important to find an efficient route for the administration of DNA vaccines into the appropriate target cells.

Intradermal administration of DNA vaccines via a gene gun is a convenient way of delivering such vaccines into professional antigen-presenting cells (APCs) in vivo. Professional APCs are the best candidate to mediate the presentation of antigens encoded by DNA vaccines to T cells. The gene gun strategy enables efficient delivery of DNA into epidermal bone marrow-derived APCs called Langerhans cells, which move into the lymphatic system via the draining lymph nodes (5). We have successfully used this system of DNA delivery to test various intracellular targeting strategies (2, 17).

One major limitation of DNA vaccines is their inability to amplify and spread in vivo as some replicating viral vaccine vectors are able to do. Therefore, a strategy that facilitates the spread of antigen may significantly enhance the potency of naked DNA vaccines. Recently, our group, as well as other researchers, has been able to enhance the potency of DNA vaccines by using human herpes simplex virus type 1 (HSV-1) (13, 25) or bovine herpesvirus 1 (24) VP22. VP22, a tegument protein that has demonstrated the property of intercellular transport, is capable of distributing protein to many surrounding cells (9). We showed that HSV-1 VP22 is capable of enhancing intercellular spreading of the linked protein, leading to augmentation of E7-specific CD8$^+$-T-cell precursor numbers and enhancement of the antitumor effect in HSV-1 VP22/E7 DNA-vaccinated mice compared with wild-type E7 DNA-vaccinated mice (13). The success of this strategy warranted the consideration of other proteins with similar trafficking properties.

Recently, a tegument phosphoprotein of Marek's disease virus serotype 1 (MDV-1) has been shown to be capable of intercellular transport after exogenous application (8). MDV-1 UL49 codes for a protein of 249 amino acids (aa) with a molecular mass of 27.6 kDa (22). In addition, the protein encoded by MDV-1 UL49 exhibits homology to HSV-1 VP22 (20). We therefore investigated the novel use of MDV-1 VP22 linked to a model antigen (human papillomavirus type 16 [HPV-16] E7) in the context of a DNA vaccine (MDV-1 VP22/E7) and explored its ability to enhance E7-specific immune responses and antitumor effects. Our data indicated that the linkage of MDV-1 VP22 to E7 also led to an impressive increase in the number of E7-specific CD8$^+$-T-cell precursors and generated significant potency against E7-expressing tumors in vaccinated mice. Mice vaccinated with MDV-1 VP22/E7 DNA generated E7-specific CD8$^+$-T-cell precursors in numbers comparable to those produced by mice vaccinated with HSV-1 VP22/E7. Our results suggested that the fusion of MDV-1 VP22 DNA to a target antigen gene could significantly enhance DNA vaccine potency and that MDV-1 VP22 represents an alternative to HSV-1 VP22 for enhancing vaccine potency.

MATERIALS AND METHODS

Plasmid DNA construction. We employed the pcDNA3 expression vector, which has been used effectively to investigate the correlation between the E7-specific T-cell-mediated immune response and the antitumor effect generated by various DNA vaccines (2). The generation of pcDNA3-E7 has been described previously (2). For the generation of pcDNA3-E7(E/B), which contains E7 with

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*Eco*RI and *Bam*HI restriction sites on the flanking ends of the E7 DNA, we first used PCR to amplify the E7 fragment with pcDNA3-E7 and a set of primers, 5′-GGGGAATTCATGGAGATACACCTA-3′ and 5′-GGTGGATCCTTGAG AACAGATGG-3′. The amplified product was further cloned into the *Eco*RI and *Bam*HI sites of pcDNA3. For the generation of pcDNA3–MDV-1 VP22, we first performed PCR to amplify the MDV-1 VP22 DNA fragment, using pUC18BE8.6, a DNA template containing MDV-1 UL49 (35) (kindly provided by Lucy F. Lee, United States Department of Agriculture, East Lansing, Mich.), and a set of primers, 5′-ATCTCTAGAATGGGGGATTCTGAAAGGCG-3′ and 5′-GATGAATTCTTCGCTATCACTGCTACGAT-3′. The amplified product was cloned into the unique *Eco*RI and *Xba*I cloning sites of the pcDNA3.1(−) expression vector (Invitrogen, Carlsbad, Calif.) downstream of the cytomegalovirus promoter. For the generation of pcDNA3–MDV-1 VP22/E7, the same PCR-amplified product (MDV-1 VP22) was cloned into the unique *Xba*I and *Eco*RI cloning sites of pcDNA3-E7(E/B). The MDV-1 VP22 gene was cloned in frame with the E7 gene. DNA sequencing confirmed that the vaccine encoded the MDV-1 VP22/E7 fusion protein.

DNA vaccination. Preparation of DNA-coated gold particles and gene gun particle-mediated DNA vaccination were performed according to previously described protocols (2). DNA-coated gold particles (1 μg of DNA/bullet) were delivered to the shaved abdominal regions of C57BL/6 mice with a helium-driven gene gun (Bio-Rad, Hercules, Calif.) with a discharge pressure of 400 lb/in^2. Mice were vaccinated via gene gun with 2 μg of MDV-1 VP22, E7, MDV-1 VP22 mixed with E7 (MDV-1 VP22+E7 DNA), MDV-1 VP22/E7, or pcDNA3 (which has no insert). One week later, mice were boosted with the same regimen as the first vaccination.

Mice. C57BL/6 mice (6- to 8-week-old females) from the National Cancer Institute (Frederick, Md.) were purchased and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, Md.). We have used these mice in previous DNA vaccine studies (2, 4, 13–15, 17). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

Intracellular cytokine staining. Cell surface marker staining of CD8 or CD4, intracellular cytokine staining for gamma interferon (IFN-γ), and FACScan analysis were performed using conditions described previously (2). Prior to FACScan analysis, splenocytes from different vaccinated groups of mice were collected 8 days after the final vaccination and incubated for 20 h with either an E7 peptide (aa 49 to 57; RAHYNIVTF) (10) containing a major histocompatibility complex (MHC) class I epitope for detecting E7-specific CD8$^+$-T-cell precursors (1 μg/ml) or an E7 peptide (aa 30 to 67; DSSEEEDEIDGPAGQA EPDRAHYNIVTFCCKCDSTLRL) (33) containing an MHC class II epitope for detecting E7-specific CD4$^+$-T-cell precursors (10 μg/ml). The numbers of IFN-γ-secreting CD8$^+$ and CD4$^+$ T cells were determined by flow cytometry.

ELISA. The levels of anti-HPV-16 E7 antibodies in sera were determined by a direct enzyme-linked immunosorbent assay (ELISA) as described previously (34). Briefly, wells of a 96-microwell plate were coated with 100 μl of a 10-μg/ml solution of bacterium-derived HPV-16 E7 protein and incubated at 4°C overnight. The wells were then blocked with phosphate-buffered saline (PBS) containing 20% fetal bovine serum. Sera were prepared from the mice on day 14 postimmunization, serially diluted in PBS, added to the ELISA wells, and incubated at 37°C for 2 h. After being washed with PBS containing 0.05% Tween 20, the plate was incubated with a 1/2,000 dilution of a peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (Zymed, San Francisco, Calif.) at room temperature for 1 h. The plate was washed six times, and then 1-Step Turbo TMB-ELISA was used as a substrate for color development (Pierce, Rockford, Ill.); color development was stopped with 1 M H_2SO_4. The absorbance of the ELISA plate wells was then determined with a standard ELISA reader at 450 nm.

In vivo tumor protection experiment. For the tumor protection experiment, mice (five per group) were vaccinated via gene gun with 2 μg of pcDNA3 (no insert), MDV-1 VP22, E7, MDV-1 VP22+E7, or MDV-1 VP22/E7 DNA. One week later, mice were boosted with the same regimen as was used in the first vaccination. One week after the last vaccination, mice were each challenged with 5×10^4 TC-1 tumor cells subcutaneously, in the right leg, and then monitored twice a week. The day of the first gene gun vaccination was considered the starting day for counting postoperative survival days.

In vivo tumor treatment experiment. The day of intravenous tumor challenge was considered the starting day for this experiment. On the starting day, mice (five per group) were each intravenously challenged with 10^4 TC-1 tumor cells via the tail vein. Three days after the first challenge, mice were administered 2 μg of pcDNA3 (no insert), MDV-1 VP22, E7, MDV-1 VP22+E7 DNA, or MDV-1 VP22/E7 DNA via gene gun. One week after the first vaccination, these mice were boosted with the same regimen. Mice were sacrificed and lungs were explanted on day 24. The pulmonary nodules on the surfaces of the lungs in each mouse were counted and weighed by researchers blinded to sample identity. Compared with the percentage of tumor-free mice, used as a measurement with the subcutaneous tumor model, the mean number of tumor nodules in the pulmonary tumor model provides a more quantitative assessment of the antitumor effect generated by each vaccine. The pulmonary tumor model might allow the evaluation of subtle differences in antitumor effects that might not be appreciable when our subcutaneous tumor model is used.

In vivo antibody depletion experiment. In vivo antibody depletion has been described previously (21). Briefly, mice were each vaccinated with 2 μg of MDV-1 VP22/E7 DNA via gene gun, boosted 1 week later, and challenged with 5×10^4 TC-1 tumor cells subcutaneously. Depletions were started 1 week prior to tumor challenge. Monoclonal antibody (MAb) GK1.5 (6) was used for CD4 depletion, MAb 2.43 (29) was used for CD8 depletion, and MAb PK136 (19) was used for NK1.1 depletion. Depletion was terminated on day 70 after tumor challenge.

RESULTS

Comparison of amino acid sequence of MDV-1 UL49 (VP22 homologue) with that of HSV-1 VP22. HSV-1 and MDV-1 are classified under the subfamily *Alphaherpesviridae*. The MDV-1 VP22 (UL49) and HSV-1 VP22 sequences were optimally aligned using the ClustalW method with a Blosum30 residue weight table as shown in Fig. 1. The results revealed approximately 25% similarity between the VP22s from HSV-1 and MDV-1, with the C-terminal halves of the sequences having a higher degree of similarity than the remaining portions of the sequences. Despite the limited degree of homology, the MDV-1 VP22 (UL49) protein has been shown to exhibit an intercellular spreading property similar to that demonstrated by HSV-1 VP22 (8).

Vaccination with MDV-1 VP22/E7 fusion DNA significantly enhances E7-specific CD8$^+$-T-cell-mediated immune responses. We have previously demonstrated that a DNA vaccine encoding HSV-1 VP22 linked to E7 generated significant enhancement of E7-specific CD8$^+$-T-cell precursors in vaccinated mice (13). To determine whether vaccination of mice with the pcDNA3–MDV-1 VP22/E7 DNA vaccine could enhance the number of E7-specific CD8$^+$-T-cell precursors, we performed intracellular cytokine staining on splenocytes from vaccinated mice. Splenocytes from naive or vaccinated groups of mice were incubated with the MHC class I (H-2D^b)-restricted E7 peptide (aa 49 to 57) for detection of E7-specific CD8$^+$ T cells. As shown in Fig. 2A, mice vaccinated with MDV-1 VP22/E7 DNA exhibited a significant increase in E7-specific IFN-γ$^+$ CD8$^+$-T-cell precursors (451 per 3×10^5 splenocytes) compared to mice vaccinated with wild-type E7 DNA (12 per 3×10^5 splenocytes). The physical linkage of MDV-1 VP22 to E7 was important for the observed enhancement of E7-specific CD8$^+$-T-cell activity since MDV-1 VP22+E7 DNA did not generate a significant increase in the number of CD8$^+$-T-cell precursors (20 per 3×10^5 splenocytes). Furthermore, the linkage of an irrelevant protein (such as green fluorescent protein or cytotoxic T-lymphocyte antigen 4) to E7 did not generate enhancement of E7-specific CD8$^+$-T-cell activity (data not shown).

While addition of MDV-1 VP22 to E7 led to enhanced E7-specific CD8$^+$-T-cell activity, we did not detect a significant difference in the number of E7-specific, IFN-γ-secreting CD4$^+$ T cells (Fig. 2B) among the various vaccination groups. Using a direct ELISA, we found that there was a slight increase in the E7-specific antibody response in mice vaccinated with E7-con-

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MDV-1 ---------------------MGDSERR--KSERR---RS----LGY-PS---AYDDVSI
HSV-1 CTCGCCCCAGACGGCCCGTCGAGMTSRRSVKSGPREVPRDEYEDLYYTPSSGMASPD-SP
      1........10........20........30........40........50.........

MDV-1 P--ARRPS--TRT-QRN---LNQ-D--DLSKHGPFT--D--HP----TQKHKSAKAVS--
HSV-1 PDTSRRGALQTRSRQRGEVRFVQYDESDYALYGGSSEDDEHPEVPRTRRPVSGAVLSGP
      61.......70........80........90........100.......110........

MDV-1 ----------------------------EDVSS---------TTRGGFTHKPRT-----K
HSV-1 GPARAPPPPAGSGGAGRTPTTAPRAPRTQRVASKAPAAPAAETTRGRKSAQPESAALPDA
      121......130.......140.......150.......160.......170........

MDV-1 PG----VR---AVQ--SNKFAFSTAPSSASSTWRSNTVAFNQHMFCGAVATVAQYHAYQG
HSV-1 PASTAPTRSKTPAQGLARKLHFSTAPPNPDAPWTPRVAGFNKRVFCAAVGRLAAMHARMA
      181......190.......200.......210.......220.......230........

MDV-1 ALALWRQDPPRTNEELDAFLSRAVIKITIQEGPNLMGEAETCARKLLEESGLSQGNENVK
HSV-1 AVQLWDMSRPRTDEDLNELLGITTIRVTVCEGKNLLQRAN----ELV--------NPDVV
      241......250.......260.......270.......280.......290........

MDV-1 SKSERTTKSERTTRRGGEIEIKSPDPGSHRTHNPRTPATSHRHHSSARGYRSSDSEZ
HSV-1 QDVDAAT---ATR--GRSAASRP---TER---PRAPA--R---SASRPRR--PVE-
      301......310.......320.......330.......340.......350....
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FIG. 1. Alignment of amino acid sequences of the VP22 tegument proteins from HSV-1 and MDV-1. Shaded regions represent matched amino acids found in at least three of the five aligned sequences. Horizontal lines represent gaps. Note that the MDV-1 VP22 and HSV-1 VP22 amino acid sequences were optimally aligned by the ClustalW(accurate) method with a Blosum30 residue weight table. The results revealed approximately 25% similarity between the MDV and HSV-1 VP22 sequences.

taining DNA vaccines compared to mice vaccinated with the empty vector (pcDNA3). We did not detect a significant difference in E7-specific antibody responses in the sera of mice vaccinated with E7, MDV-1 VP22+E7, or MDV-1/E7 DNA (Fig. 2C).

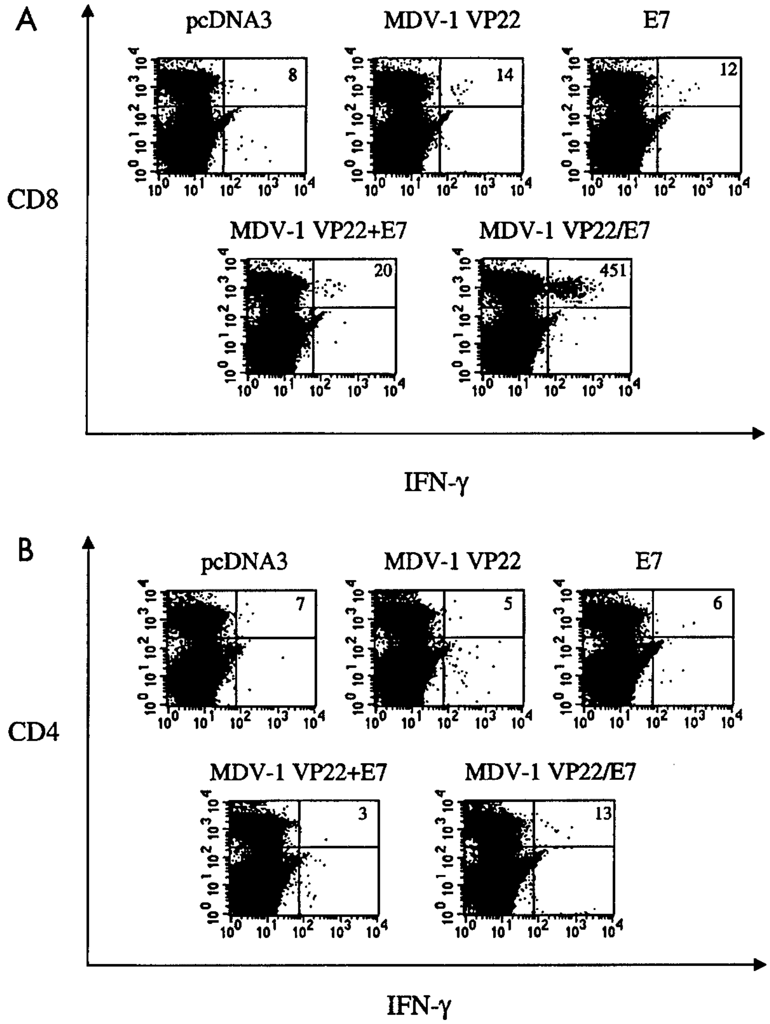
Vaccination with a chimeric MDV-1 VP22/E7 DNA vaccine enhances protection of mice against growth of TC-1 tumors. To determine whether the observed enhancement of E7-specific $CD8^+$-T-cell-mediated immunity translated to a significant E7-specific antitumor effect, we performed an in vivo tumor protection experiment using a previously characterized E7-expressing tumor model, TC-1 (21). As shown in Fig. 3, 100% of mice receiving the MDV-1 VP22/E7 DNA vaccine remained tumor free 65 days after TC-1 challenge. In contrast, all of the mice receiving pcDNA3 (no insert), wild-type E7, or MDV-1 VP22+E7 DNA developed tumors within 21 days after tumor challenge. We also observed that fusion of E7 to MDV-1 VP22 was required for antitumor immunity, since MDV-1 VP22+E7 DNA did not generate enhancement of tumor protection.

Treatment with MDV-1 VP22/E7 fusion DNA eradicates established E7-expressing tumors in the lungs. We then investigated the therapeutic potential of the chimeric MDV-1 VP22/E7 DNA construct in treating TC-1 tumors, using a lung hematogenous spread model (16). As shown in Fig. 4A, mice vaccinated with MDV-1 VP22/E7 DNA exhibited fewer pulmonary nodules (mean ± standard error of the mean, 3.2 ± 1.9) than mice vaccinated with wild-type E7 DNA (74.2 ± 11.27) or MDV-1 VP22 DNA (86.6 ± 12.5). Also, mice vaccinated with MDV-1 VP22/E7 DNA exhibited a lower average lung weight than mice vaccinated with wild-type E7 DNA or MDV-1 VP22 DNA (data not shown). Taken together, the results from our tumor protection and treatment experiments indicated that linkage of MDV-1 VP22 to E7 significantly enhanced antitumor effects on TC-1 tumors.

$CD8^+$ T cells, but not $CD4^+$ T cells or NK cells, are important for antitumor effect generated by the MDV-1 VP22/E7 DNA vaccine. To determine the subset of lymphocytes that is important for the antitumor effect generated by the MDV-1 VP22/E7 DNA vaccine, we performed in vivo antibody depletion experiments. As shown in Fig. 5, all MDV-1 VP22/E7 DNA-vaccinated mice depleted of $CD8^+$ T cells and all unvaccinated naive mice grew tumors within 14 days after tumor challenge. In contrast, all MDV-1 VP22/E7 DNA-vaccinated mice depleted of $CD4^+$ T cells and NK cells remained tumor free 70 days after tumor challenge. These results suggested that $CD8^+$ T cells, but not $CD4^+$ T cells or NK cells, were essential for the antitumor immunity generated by the VP22/E7 DNA vaccine.

DISCUSSION

In this study, we observed that MDV-1 VP22 enhanced DNA vaccine potency when linked to a model antigen, E7. Despite the limited similarity between the amino acid sequences of MDV-1 UL49 (VP22) and HSV-1 VP22 (approximately 25%), the MDV-1 VP22/E7 and HSV-1 VP22/E7 (13) DNA vaccines generated comparable degrees of E7-specific $CD8^+$-T-cell-mediated immune response. The property of intercellular spreading shared by these VP22 homologues may differ from that of other molecules with trafficking potential. We recently observed that DNA vaccines encoding E7 linked to molecules derived from other proteins with trafficking properties (such as human immunodeficiency virus Tat protein, membrane-translocating sequence, and the third helix of the Antennapedia homeodomain in *Drosophila*) did not generate



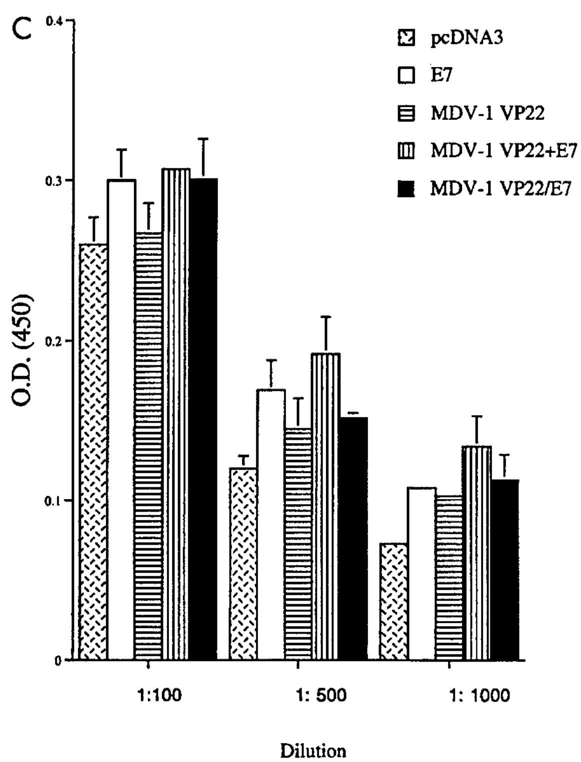


FIG. 2. Immunologic responses generated by various recombinant DNA vaccines. (A) Results of flow cytometry analysis of IFN-γ-secreting, E7-specific CD8$^+$-T-cell precursors. Mice were immunized and splenocytes were collected and cultured as described in Materials and Methods. Note that vaccination of mice with MDV-1 VP22/E7 DNA generated a larger number of IFN-γ$^+$ CD8$^+$-T-cell precursors than did vaccination with any of the other constructs. The results shown are from one representative experiment of three that were performed. (B) Results of flow cytometry analysis of IFN-γ-secreting, E7-specific CD4$^+$-T-cell precursors. (C) Results of an ELISA to determine E7-specific antibodies in the sera of the various vaccinated mice. Mice were immunized and sera were collected and cultured as described in Materials and Methods. O.D. (450), optical density at 450 nm.

CD8$^+$-T-cell-mediated immune responses as potent as those generated by HSV-1 VP22/E7 (13) or MDV-1 VP22/E7.

Alignment of the amino acid sequences of MDV-1 VP22 and HSV-1 VP22 revealed that the carboxyl-terminal halves of the sequences exhibit a considerably higher degree of similarity than the amino-terminal halves, suggesting that the C-terminus region may be important for VP22 function. Studies have shown that the C-terminus region is important for intercellular spreading (9) and nuclear localization (27). Deletion mutants linked to fluorescent proteins might be used to identify the precise region of MDV-1 VP22 that is responsible for intercellular spreading. Recently, HSV-1 VP22's cytoskeleton binding, nuclear localization, chromatin binding, nuclear mem-

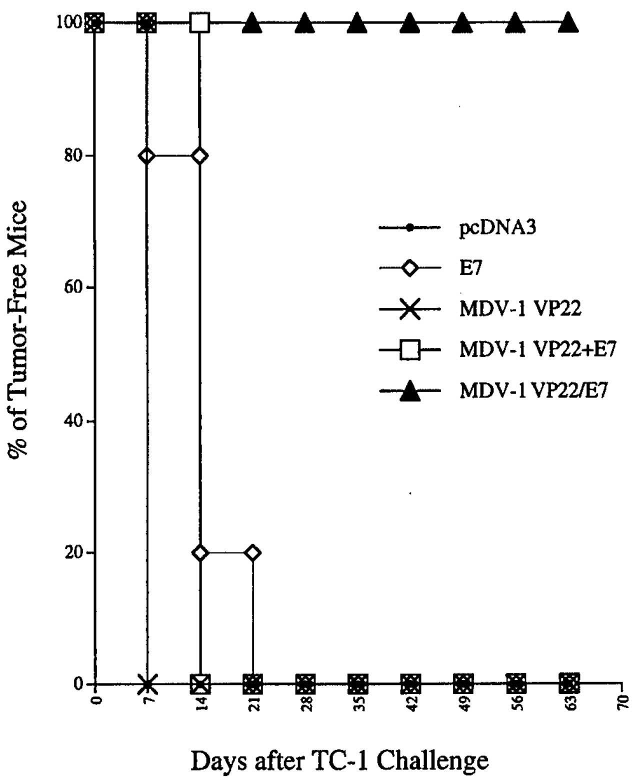


FIG. 3. Tumor protection experiments and the effect of MDV-1 VP22/E7 on subcutaneous tumor cell growth. In the in vivo tumor protection experiments, mice were immunized and challenged subcutaneously with TC-1 tumor cells as described in Materials and Methods. The data shown here are from one representative experiment of two that were performed.

brane binding, and intercellular transport domains were mapped using such deletion experiments (1). Mapping the functional domains that are important for the ability of MDV-1 VP22 and other homologues to enhance vaccine potency would be an important endeavor for the future.

We found that vaccination with MDV-1 VP22/E7 generated an immune response and antitumor effect comparable to those provided by HSV-1 VP22/E7 DNA, although a more definitive conclusion requires a head-to-head comparison. Meanwhile, a notable difference between the MDV-1 VP22/E7 and HSV-1 VP22/E7 DNA vaccines in terms of the effector cells responsible for antitumor immunity was observed. While $CD8^+$ T cells and NK cells were found to be important for the antitumor effect generated by HSV-1 VP22/E7 DNA (13), our data revealed that the antitumor effect generated by MDV-1 VP22/E7 DNA required only $CD8^+$ T cells. These data imply that the two vaccines may activate different subsets of effector cells in the vaccinated host and may have different immunologic or antitumor mechanisms. It would be interesting to perform additional experiments to evaluate qualitative and/or quantitative differences in NK cells in mice vaccinated with MDV-1 VP22 or its homologues.

Several factors may account for the observed enhancement of E7-specific $CD8^+$-T-cell activity in mice administered DNA vaccines encoding MDV-1 VP22/E7 or other VP22 homologues linked to E7. First, since ballistic DNA delivery can introduce DNA directly into dermal professional APCs, the intercellular spreading of the MDV-1 VP22 and HSV-1 VP22 protein within the epidermis raises the possibility of generating an increased number of APCs that present the linked protein. This notion is supported by our previous study using an HSV-1 VP22 deletion mutant, HSV-1 VP22 (aa1–267), which lacks the property of intercellular spreading (13). Linkage of HSV-1 VP22 (aa1–267) to antigen fails to enhance DNA vaccine potency. Second, we have previously shown that cells transfected with DNA encoding HSV-1 VP22/E7 directly enhance the presentation of E7 through the MHC class I pathway to an E7-specific $CD8^+$-T-cell line (13), suggesting that direct priming may be an important mechanism for enhancing E7-specific $CD8^+$-T-cell activity. Third, we have demonstrated that dendritic cells pulsed with lysates of cells transfected with naked HSV-1 VP22/E7 RNA replicons exhibit a significantly higher percentage of specific lysis by an E7-specific $CD8^+$-T-cell line than dendritic cells pulsed with lysates of cells transfected with wild-type E7 RNA replicons (3), indicating that cross-priming may be another potential mechanism for the enhancement of E7-specific $CD8^+$-T-cell responses mediated by VP22/E7. Finally, DNA vaccines may influence the biology of professional APCs at vaccination sites through the effects of CpG islands (18, 30, 32) or induced cytokines (12, 18), which in turn may affect vaccine-mediated, antigen-specific $CD8^+$-T-cell activity.

We have previously developed several intracellular targeting strategies that are capable of enhancing MHC class I and class

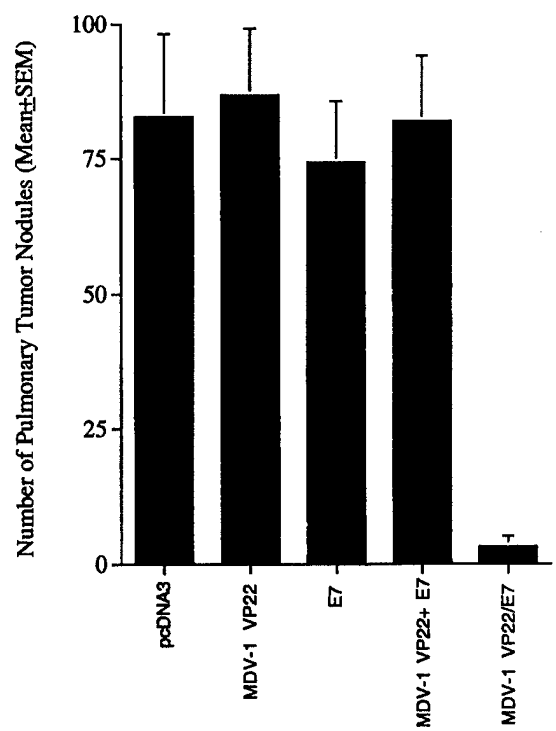


FIG. 4. Tumor treatment experiments and the effect of MDV-1 VP22/E7 on pulmonary tumor cell growth. The mean number of lung tumor nodules generated in each vaccinated group was determined. For the in vivo tumor treatment experiments, mice (five per group) were challenged intravenously in the tail vein and vaccinated as described in Materials and Methods.

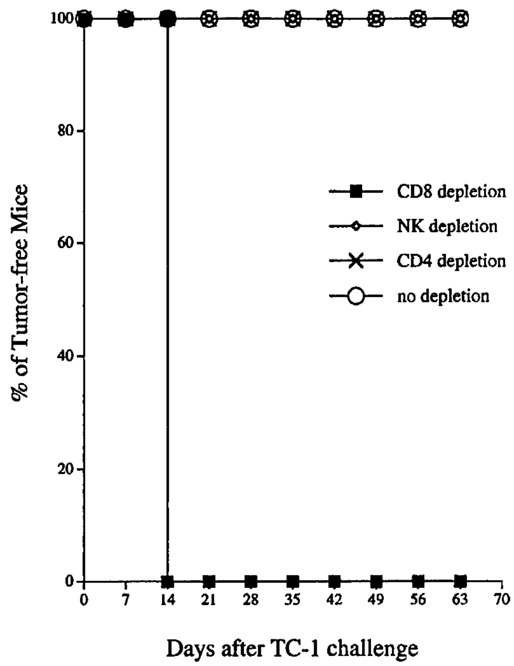


FIG. 5. In vivo antibody depletion experiment. An in vivo antibody depletion experiment was performed to determine the effect of lymphocyte subsets on the potency of the MDV-1 VP22/E7 DNA vaccine. Mice were vaccinated, challenged with TC-1, and depleted of the relevant subset of lymphocytes as described in Materials and Methods. Note that depletion of $CD8^+$ T cells, but not of $CD4^+$ T cells or NK cells, led to significant tumor growth in MDV-1 VP22/E7 DNA-vaccinated mice.

II presentation of antigens encoded by DNA vaccines. For example, an endosomal-lysosomal targeting strategy using E7 chimerically linked to a signal peptide (Sig) and the endosomal-lysosomal sorting signal (derived from lysosome-associated membrane protein 1) was found to significantly enhance MHC class II presentation of antigen to $CD4^+$ T cells (17). Furthermore, we have used several different molecules, including *Mycobacterium tuberculosis* heat shock protein 70 (2), domain II of *Pseudomonas* exotoxin A (14), and calreticulin (4), linked to antigen in the context of DNA vaccines to significantly enhance MHC class I presentation of the E7 antigen to $CD8^+$ T cells. The vaccine potency of these intracellular approaches may be further enhanced if the intercellular spreading strategy is also incorporated into the vaccine design.

Safety is an important issue to address before considering broader applications of this vaccine. Although DNA may integrate into the host genome, it is estimated that the frequency of integration is much lower than that of spontaneous mutation and should not pose any real risk (23). Potential risks associated with the presence of the HPV-16 oncogenic E7 protein in host cells may be alleviated by introducing mutations into E7 DNA so that the resulting E7 protein cannot bind with pRB (11) but still maintains most of its antigenicity. Another strategy is to use a shuffled E7 gene, which can alleviate concerns of oncogenicity associated with E7 (25). Another concern about DNA vaccination is the generation of autoimmune disease against host DNA. However, we examined vital organs in MDV-1 VP22/E7-vaccinated mice and did not observe any significant pathological changes. These results indicated that MDV-1 VP22/E7 could be an effective DNA vaccine with minimal detrimental side effects.

In summary, our results indicated that the fusion of MDV-1 VP22 to the HPV-16 E7 gene generated powerful E7-specific $CD8^+$-T-cell-mediated immune responses and antitumor effects against HPV-16 E7-expressing murine tumors. The VP22 strategy can potentially be applied to other cancer systems with known tumor-specific antigens. It would be valuable to compare VP22 homologues and determine whether the extent of intercellular spreading of linked antigen encoded by DNA vaccine can be correlated with the degree of antigen-specific immunity and the ensuing antitumor effect.

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